Extrinsic cues, intrinsic cues and microfilaments regulate asymmetric protein localization in *Drosophila* neuroblasts

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Background: The *Drosophila* central nervous system develops from stem cell like precursors called neuroblasts, which divide unequally to bud off a series of smaller daughter cells called ganglion mother cells. Neuroblasts show cell-cycle-specific asymmetric localization of both RNA and proteins: at late interphase, *prospero* RNA and Inscuteable, Prospero and Staufen proteins are all apically localized; at mitosis, Inscuteable remains apical whereas *prospero* RNA, Prospero protein and Staufen protein form basal cortical crescents. Here we use *in vitro* culture of neuroblasts to investigate the role of intrinsic and extrinsic cues and the cytoskeleton in asymmetric localization of Inscuteable, Prospero and Staufen proteins.

Results: Neuroblast cytokinesis is normal *in vitro*, producing a larger neuroblast and a smaller ganglion mother cell. Apical localization of Inscuteable, Prospero and Staufen in interphase neuroblasts is reduced or eliminated *in vitro*, but all three proteins are localized normally during mitosis (apical Inscuteable, basal Prospero and Staufen). Microfilament inhibitors result in delocalization of all three proteins. Inscuteable becomes uniform at the cortex, whereas Prospero and Staufen become cytoplasmic; inhibitor washout leads to recovery of microfilaments and asymmetric localization of all three proteins. Microtubule disruption has no effect on protein localization, but disruption of both microtubules and microfilaments results in cytoplasmic localization of Inscuteable.

Conclusions: Both extrinsic and intrinsic cues regulate protein localization in neuroblasts. Microfilaments, but not microtubules, are essential for asymmetric protein anchoring (and possibly localization) in mitotic neuroblasts. Our results highlight the similarity between *Drosophila*, *Caenorhabditis elegans*, vertebrates, plants and yeast: in all organisms, asymmetric protein or RNA localization and/or anchoring requires microfilaments.

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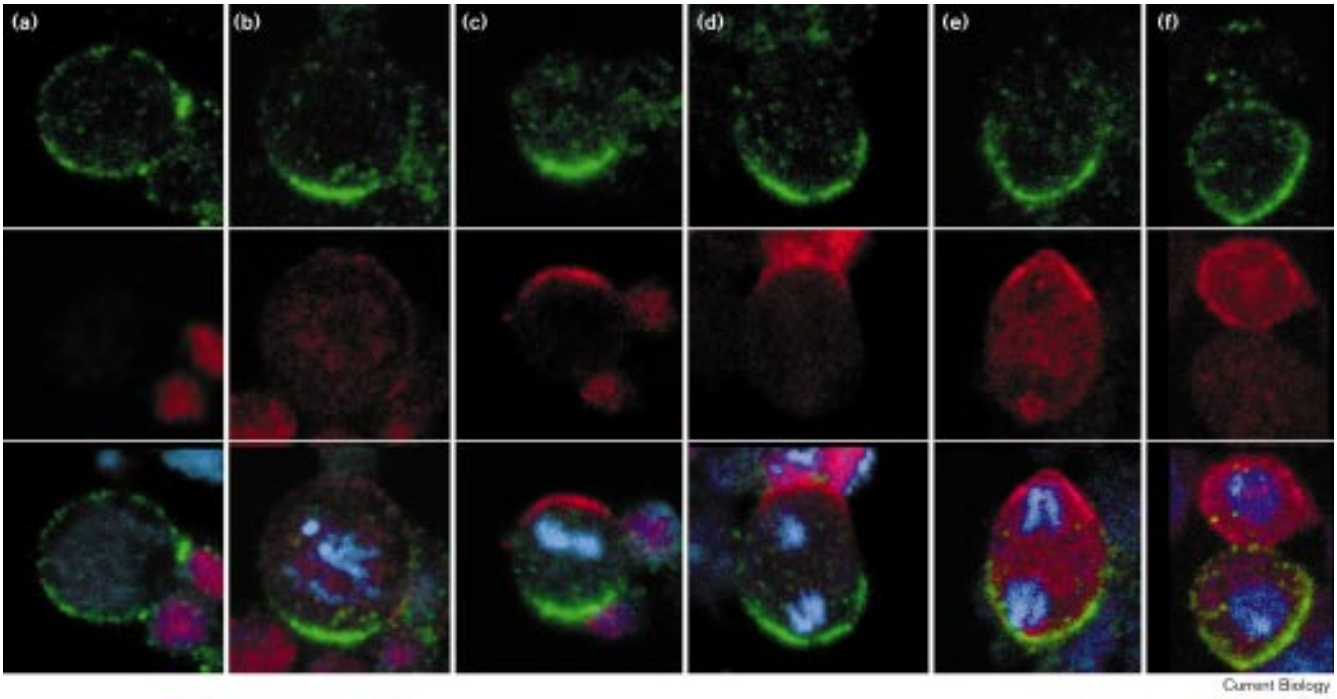
Background

Asymmetric protein localization is often required for the proper function of differentiated cells [1], and is also used to specify sibling cell fates in organisms as diverse as *Bacillus subtilis* [2], *Caulobacter* [3], *Saccharomyces cerevisiae* [4–6], *C. elegans* [7–9], and *Drosophila* [10–12]. Recently, the *Drosophila* central nervous system (CNS) has emerged as a model system for studying asymmetric cell division and protein localization [10–17].

The *Drosophila* CNS develops from stem cell-like neuroblasts that delaminate into the embryo from an apical–basal polarized epithelium called the neuroectoderm. Neuroblasts divide along the apical–basal axis to bud off a smaller, basal daughter cell (ganglion mother cell, GMC) and regenerate a larger, apical daughter neuroblast that remains adjacent to the neuroectoderm [18,19]. During neuroblast mitosis, several proteins and one RNA are localized to the basal cortex and partitioned into the GMC: *prospero* (*pros*) RNA and Pros protein, Staufen (Stau) protein, Miranda (Mira) protein and Numb protein ([13–15,20–23]; J.B., S. Fuerstenberg, C.Q.D., unpublished observations). The Pros transcription factor is translocated into the GMC nucleus and is required for GMC development [13,24]; Numb protein remains at the GMC cortex and has no identified function in the GMC [13–15]; the RNA-binding Stau protein is required for *pros* RNA localization ([23]; J.B., S. Fuerstenberg, C.Q.D., unpublished observations), and the membrane-associated Mira protein is required for Pros basal localization [20,21]. In addition, localization of all basal proteins (Pros, Numb, Mira and Stau) requires the function of Inscuteable (Insc), which is associated with the apical cortex of the neuroblast from late interphase through mitosis [16,17]. Both Pros and Stau have transient apical co-localization with Insc during interphase ([23]; J.B., S. Fuerstenberg, C.Q.D., unpublished observations), although this does not seem to be necessary for their subsequent basal localization at mitosis as we describe here.

Figure 1



Cell-cycle-dependent asymmetric localization of Insc and Pros in neuroblasts *in vitro*. Neuroblasts grown *in vitro* and triple-labeled for Insc (green, top row), Pros (red, middle row), and DNA (blue, bottom row, merged with Insc and Pros); the same neuroblast is pictured in each column. Note that apical is down, basal is up (see Materials and methods for details). **(a)** During late interphase, Insc is detected uniformly at the neuroblast cortex; Pros is undetectable. **(b)** During prophase, Insc forms an apical cortical crescent; Pros is weakly cortical and nuclear or undetectable. At metaphase **(c)**, anaphase **(d)** and telophase **(e)**, Insc and Pros are localized as cortical crescents on opposite sides of the neuroblast; there is a clear gap between the apical Insc crescent and the basal Pros crescent. **(f)** Following cytokinesis, Insc is segregated to the apical neuroblast and Pros is segregated to the basal GMC. GMCs can contain Insc *in vitro* (f) and *in vivo* [16], presumably because of *de novo* production of Insc in the GMC.

We are interested in the mechanisms controlling asymmetric cell division and protein localization in *Drosophila* neuroblasts. What is the role of intrinsic versus extrinsic cues in establishing asymmetry? What is the role of the cytoskeleton in establishing asymmetry? Primary culture of embryonic neuroblasts has previously been used to study neuroblast cell lineage and neuronal differentiation *in vitro* [21–26]; here we use primary cell culture of neuroblasts to determine the contribution of extrinsic and intrinsic signals in controlling unequal neuroblast division and asymmetric localization of Insc (the earliest indicator of neuroblast polarity) and the Pros and Stau proteins. The *in vitro* culture system also allows us to use cytoskeletal inhibitors to test the role of microfilaments and microtubules in the localization and anchoring of Insc, Pros and Stau.

Results and discussion

Extrinsic cues are required for apical localization of Insc, Pros and Stau in interphase neuroblasts

Insc, Pros and Stau proteins are all asymmetrically localized to the apical cortex of neuroblasts at late interphase in wild-type embryos ([13,16,17,23]; J.B., S. Fuerstenberg, C.Q.D., unpublished observations). In contrast, late interphase neuroblasts cultured *in vitro* do not exhibit the normal strong apical crescents of Insc, Pros or Stau (see Materials and methods for the criteria used to identify the apical–basal axis in neuroblasts *in vitro*). Insc is usually undetectable, but a few neuroblasts show uniform cortical protein (Figure 1a) or very weak crescents (data not shown). Pros is also undetectable (Figure 1a) or, occasionally, detectable at low levels around the cell cortex and in the nucleus (Figure 2a). Stau is usually cytoplasmic (data not shown), but occasionally it is also weakly enriched at the cortex at one side of the neuroblast (Figure 2a). For all three proteins, we have never observed strong crescents in interphase neuroblasts *in vitro*, demonstrating that extrinsic cues are required for normal apical localization at interphase. This is true even when interphase neuroblasts are contained in high-density cell clusters (data not shown). Thus, cell contact alone is insufficient for inducing apical localization of Insc, Pros or Stau during interphase.

Within the embryo, the apical side of each neuroblast lies adjacent to the extracellular matrix (ECM) secreted

from the basal surface of the neuroectodermal epithelium; it is possible that only interaction with ECM or neuroectoderm can induce apical localization of Insc, Pros and Stau. It remains possible, however, that the *in vitro* system simply does not adequately mimic *in vivo* development in a more general manner, resulting in lack of apical protein localization. In any case, because neuroblasts show normal localization of Insc, Pros and Stau during mitosis (see below), it appears that apical localization at interphase is not a prerequisite for the subsequent localization of Insc, Pros and Stau during mitosis.

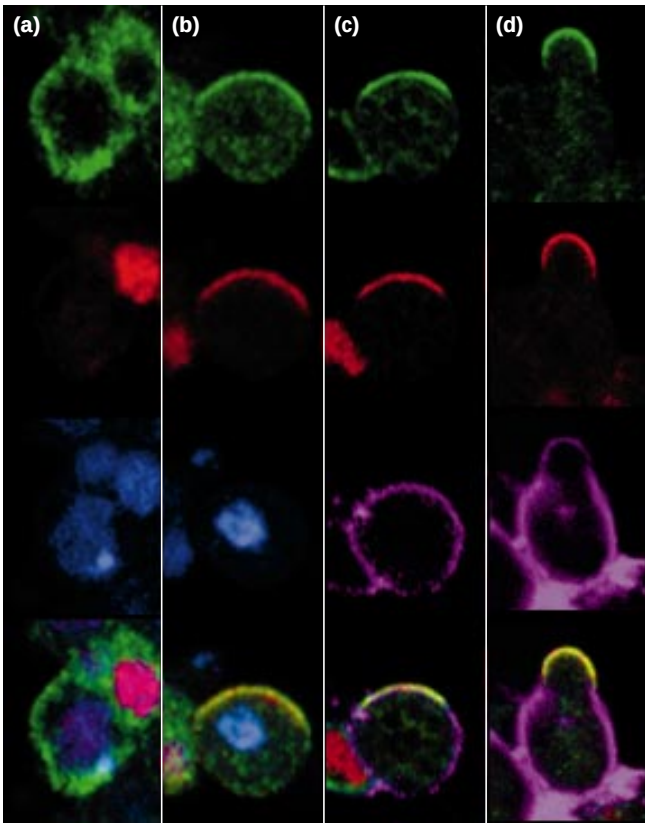
Intrinsic cues are sufficient for unequal cytokinesis and localization of Insc, Pros and Stau in mitotic neuroblasts

Neuroblasts *in vivo* and *in vitro* undergo unequal cytokinesis to produce a smaller daughter GMC and a larger neuroblast [25–30] (Figure 1). Thus, the physical asymmetry of *Drosophila* neuroblast cell division is normal in the absence of extrinsic cues. This is in contrast to grasshopper neuroblasts, which require signals from an apically associated cap cell to divide unequally *in vitro* [31].

In wild-type *Drosophila* embryos, mitotic neuroblasts maintain apical localization of Insc until the end of metaphase, at which time it appears to be degraded or delocalized [16,17]. Neuroblasts cultured *in vitro* show normal apical localization of Insc at prophase and metaphase (similarly to neuroblasts *in vivo*), but apical localization persists through anaphase and telophase (Figure 1b–e), and at cytokinesis Insc is selectively inherited by the neuroblast (Figure 1f). By mid-interphase Insc is at low levels in the cytoplasm or is undetectable (data not shown). Insc localization beyond metaphase was not observed *in vivo* [16,17]. The persistent Insc apical localization observed *in vitro* is probably due to the increased sensitivity of antibody detection *in vitro*, which we have noticed for all antibodies tested. Alternatively, it could be due to the absence of extrinsic cues that downregulate Insc levels *in vivo*. Our results show that intrinsic cues are sufficient for apical Insc localization during neuroblast mitosis. The initial establishment of neuroblast polarity is also likely to be due to intrinsic cues, inherited from the neuroectoderm, because asymmetric Insc localization can be detected in delaminating neuroblasts. One unresolved issue is the site of Insc localization in neuroblasts isolated *in vitro*: the Insc crescent may form at an apical site established in the embryo and be maintained throughout multiple cell cycles *in vitro*, or it may form at random positions at every cell cycle and organize all subsequent aspects of apical–basal polarity (e.g. Pros and Stau localization, see below).

In wild-type embryos, mitotic neuroblasts localize Pros and Stau to the basal cortex from metaphase through telophase, and both proteins are ultimately inherited by the GMC [13]. In the GMC, Pros moves into the nucleus and Stau fills the cytoplasm. Neuroblasts cultured *in vitro* show the identical asymmetric localization of Pros and Stau during mitosis. From metaphase to telophase, both Pros and Stau are localized to the basal cortex, opposite the Insc crescent (Figures 1c–e, 2b–d); following cytokinesis they are both segregated into the daughter GMC, where Pros moves into the nucleus (Figures 1f, 2a) and Stau becomes cytoplasmic ([23]; J.B., S. Fuerstenberg, C.Q.D., unpublished observations). Our results show that

Figure 2



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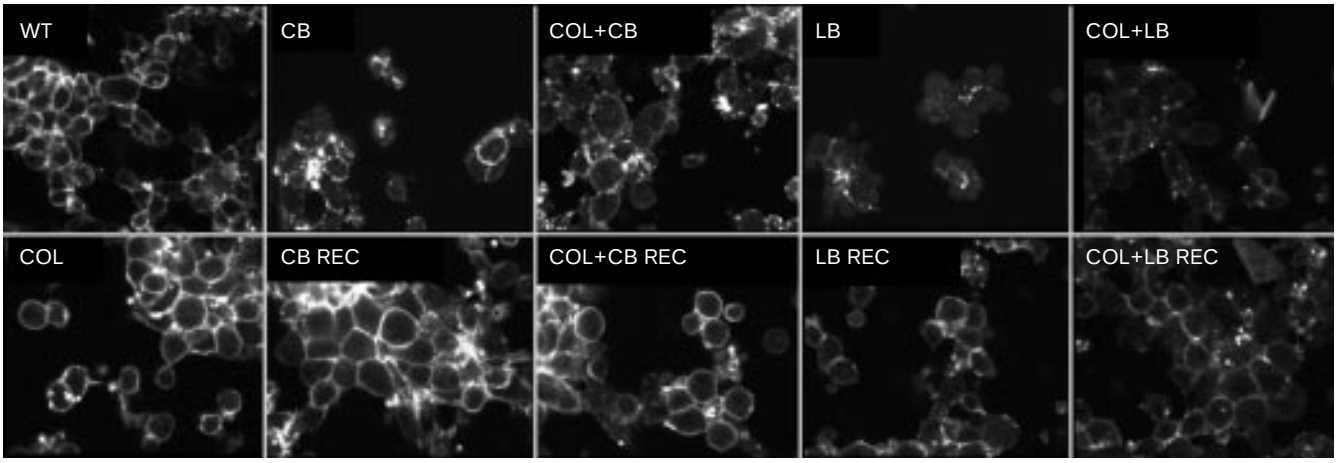
Cell-cycle-dependent asymmetric localization of Stau and Pros in neuroblasts *in vitro*. (a,b) Neuroblasts cultured *in vitro* triple-labeled for Stau (green, first row), Pros (red, second row), and DNA (blue, third row); a merged image of all three is shown in the bottom row. (c,d) Neuroblasts cultured *in vitro* triple-labeled for Stau (green, first row), Pros (red, second row), and microfilaments (purple, third row); a merged image of all three is shown in the bottom row. The same neuroblast is pictured in each column. Apical is down, basal is up (see Materials and methods for details). During interphase (a), Stau is usually detected uniformly at the neuroblast cortex or occasionally as a weak cortical crescent; Pros is undetectable or at low levels in the nucleus and at the cortex. The smaller cell at the upper right in (a) is a GMC containing nuclear Pros and cytoplasmic Stau. At metaphase (b,c), Stau and Pros are precisely co-localized as basal cortical crescents that overlap with the uniform cortical distribution of microfilaments. When Pros and Stau are at the cortex, they are always tightly co-localized (within the level of resolution provided by confocal microscopy). At anaphase (d), Stau and Pros basal crescents are restricted to the budding GMC and overlap with the uniform cortical distribution of microfilaments. The less intense microfilament staining in the budding GMC is not reproducibly observed.

Figure 3



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The effect of colcemid, cytochalasin B and latrunculin B on microfilaments *in vitro*. *In vitro* primary cell cultures labeled for microfilaments using phalloidin-FITC. See Materials and methods for drug treatment conditions. WT, untreated cells showing uniform cortical microfilaments. COL, colcemid treatment has no effect on cortical microfilaments. CB, cytochalasin B treatment transforms microfilaments into punctate spots of F-actin. CB REC, cytochalasin B treatment followed by drug washout results in total recovery of cortical microfilaments. COL+CB, colcemid plus cytochalasin B treatment transforms microfilaments into punctate spots of F-actin. COL+CB REC, colcemid plus cytochalasin B treatment followed by washout of only cytochalasin B results in total recovery of cortical microfilaments. LB, latrunculin B treatment largely eliminates microfilaments. LB REC, latrunculin B treatment followed by drug washout results in partial recovery of cortical microfilaments (note gaps at the cortex of most cells). COL+LB, colcemid plus latrunculin B treatment essentially eliminates microfilaments. COL+LB REC, colcemid plus latrunculin B treatment followed by washout of only latrunculin B results in partial recovery of cortical microfilaments.

intrinsic cues are sufficient for basal Pros and Stau localization during neuroblast mitosis. Insc is localized earlier in the cell cycle than are Pros and Stau, and thus it is likely that apically localized Insc regulates the subsequent basal localization of Pros and Stau *in vitro*, just as it controls the basal localization of Pros *in vivo* [25].

Taken together, our data on neuroblast cytokinesis and localization of Insc, Pros and Stau *in vitro* suggest that extrinsic cues regulate interphase protein localization, but that intrinsic mechanisms control asymmetric cell division and protein localization during neuroblast mitosis. Moreover, high-level apical localization of Insc, Pros and Stau during interphase is not a prerequisite for their subsequent localization during mitosis.

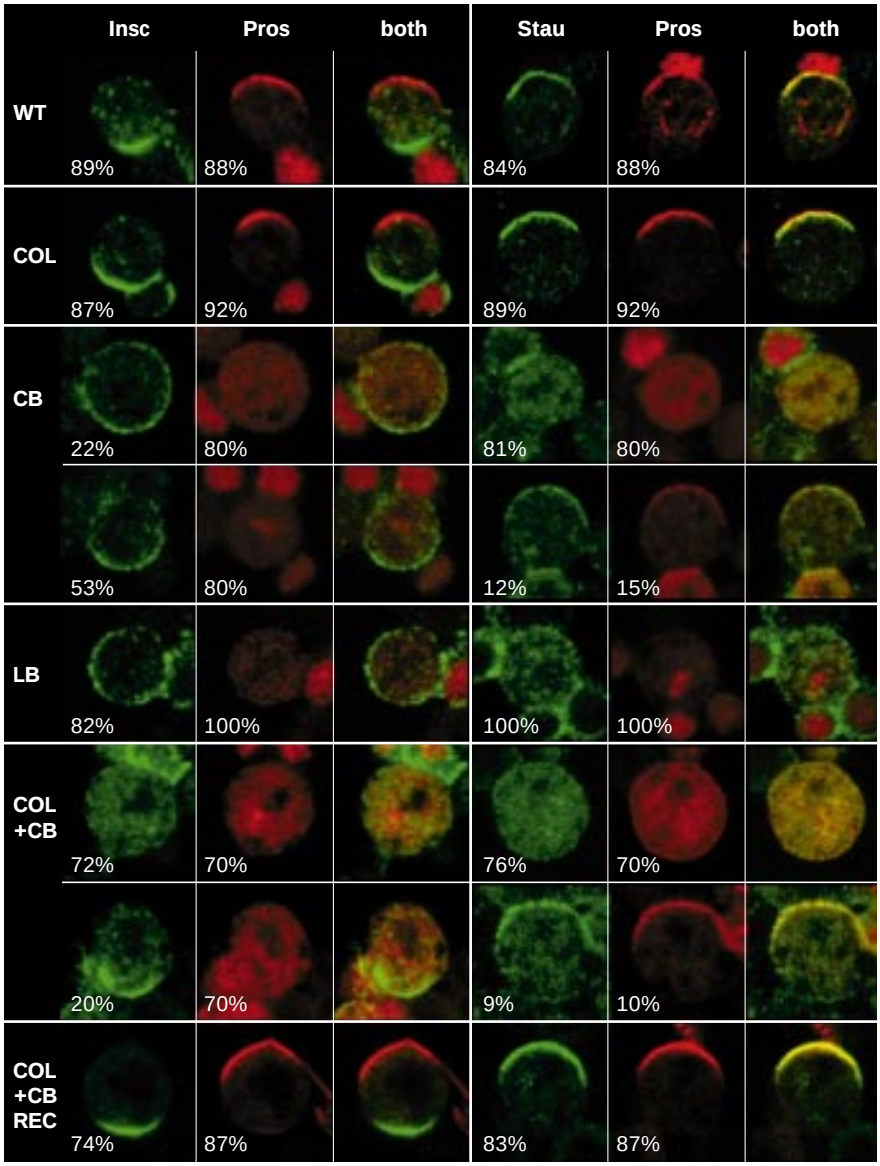
Asymmetric localization of Insc, Pros and Stau requires microfilaments but not microtubules

Drug treatments of *Drosophila* embryos *in vivo* are difficult to perform and interpret because of poor accessibility of the embryo to the drug, the inability of estimating drug levels within the embryo, and the difficulty of washing out drugs for recovery experiments. To circumvent these problems, we have applied cytoskeletal inhibitors to neuroblasts cultured *in vitro*, where we can precisely measure the dosage and time of treatment, and wash out the drugs for recovery experiments (Figures 3, 4).

Microtubules of the mitotic spindle are aligned along the apical–basal axis of dividing neuroblasts. To determine whether microtubules are involved in apical–basal protein localization in neuroblasts cultured *in vitro*, we used colcemid to disrupt microtubules and scored mitotic neuroblasts for Insc, Pros and Stau localization. Colcemid-treated neuroblasts lack microtubules (visualized following detergent extraction to remove background staining from unpolymerized tubulin; data not shown) and arrest in metaphase (Figure 4), yet there are normal apical Insc and basal Pros and Stau crescents (Figure 4). The percentage of all neuroblasts with basal Pros crescents increases as the length of colcemid treatment is increased (0 h, 7% crescents, $n = 64$; 1 h, 48.6% crescents, $n = 356$; 2 h, 92%, $n = 131$), indicating that new crescents can be generated in the absence of microtubules. We conclude that microtubules are not required for the localization or anchoring of apical Insc or basal Pros and Stau, confirming and extending previous studies on Insc [17] and Pros [13,14] localization *in vivo*. In addition, we conclude that exit from mitosis is necessary for the delocalization of Insc, Pros and Stau from the cortex in neuroblasts or GMCs (Figure 5).

Microfilaments are enriched at the cell cortex of neuroblasts, but do not have an obvious polarized distribution along the apical–basal axis *in vitro* (Figure 2c,d) or *in*

Figure 4



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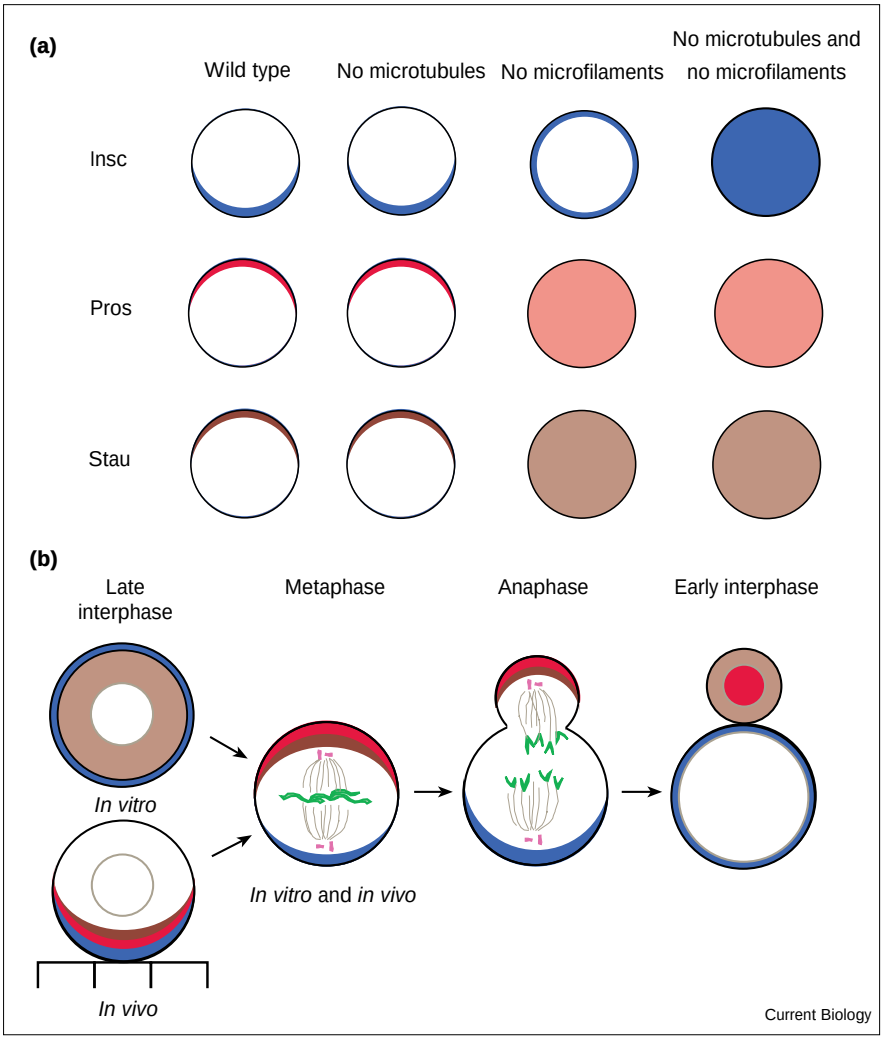
The effect of cytoskeletal inhibitors on asymmetric protein localization in metaphase neuroblasts *in vitro*. The three columns on the left show cultured neuroblasts labeled for (left to right) Insc (green), Pros (red) and the merged image; the three columns on the right show cultured neuroblasts labeled for (left to right) Stau (green), Pros (red) and the merged image. All neuroblasts are at metaphase, as determined by DNA staining (not shown). Each half row shows the same neuroblast stained for two proteins and merged. Apical is down, basal is up. See Materials and methods for details of drug treatment conditions. WT, neuroblasts show asymmetric Insc, Pros and Stau localization. COL, colcemid treatment does not affect asymmetric Insc, Pros and Stau localization. CB, cytochalasin B treatment produces some Insc crescents (bottom row), some delocalized cortical Insc (top row) and cytoplasmic Pros and Stau. LB, latrunculin B treatment produces delocalized cortical Insc and cytoplasmic Pros and Stau. COL+CB, colcemid treatment followed by cytochalasin B addition produces mostly cytoplasmic Insc, Pros and Stau (top row), although a few Insc crescents can be observed; COL+CB REC, exactly as COL+CB treatment, but then cytochalasin B is washed out: recovery of asymmetric Insc, Pros and Stau crescents is observed. Percentages indicate the frequency that the localization pattern shown in each panel was observed in all neuroblasts of the experiment; see Table 1.

vivo [13–15]. To determine whether microfilaments are involved in the localization or anchoring of Insc, Pros or Stau, we used cytochalasin B or latrunculin B to disrupt microfilaments in dividing neuroblasts. Cytochalasin B treatment at 5 μg ml^{-1} for 30 min results in punctate spots of F-actin at the cortex (Figure 3); we used this relatively high concentration and long incubation time because the effect was reversible (see below) and we wanted to maximally disrupt microfilaments. Latrunculin B treatment at 1 μg ml^{-1} for 5 min virtually eliminates F-actin staining (Figure 3); similar results were obtained using latrunculin A (data not shown). These data are consistent with previous reports showing latrunculins to be more effective microfilament inhibitors than cytochalasins [32–34]. Both cytochalasin B and latrunculin B block neuroblast cytokinesis at the concentrations used (data not shown).

We treated neuroblasts *in vitro* with either cytochalasin B or latrunculin B and scored mitotic neuroblasts for asymmetric protein localization. This treatment could perturb protein localization (if neuroblasts enter mitosis during treatment) or protein anchoring. Treatment with the weaker inhibitor, cytochalasin B, for 30 min results in partial disruption of microfilaments (Figure 3) and aberrant localization of all three proteins (Figure 4, Table 1). Insc forms apical crescents in 53% of the neuroblasts, but is also frequently delocalized around the cortex (25%) or into the cytoplasm (22%). Pros and Stau are more sensitive

Figure 5



Summary of Insc, Pros and Stau localization in neuroblasts *in vitro*. **(a)** Localization of Insc, Pros and Stau in metaphase neuroblasts *in vitro* following no drug treatment (wild type), or after disruption of the indicated cytoskeletal structures. **(b)** Cell-cycle-specific localization and regulation of Insc, Pros and Stau *in vitro* and *in vivo*. Large circle, neuroblast; small circle, GMC; partial rectangles, neuroectodermal cells. In both (a) and (b) apical is down, basal is up; Pros, red; Stau, brown; Insc, blue; DNA, green; centrosomes, pink [in (b)]. See text for details.

to partial microfilament disruption, with only 10–12% of the neuroblasts showing asymmetric localization. Insc is required for normal Pros localization [17], but we believe that loss of microfilaments directly affects Pros and Stau localization because Pros and Stau can be delocalized in neuroblasts with normal Insc crescents (Figure 4). Treatment with the more potent microfilament inhibitor latrunculin B for just 5 min produces a complete disruption of microfilaments and virtually all mitotic neuroblasts show uniform cortical localization of Insc and cytoplasmic distribution of Pros and Stau (Figure 4, Table 1). We conclude that Pros and Stau require microfilaments for localization or anchoring to the basal cortex. Moreover, microfilaments are required to restrict or anchor Insc to an apical site, because in the absence of microfilaments Insc becomes uniformly distributed around the cortex.

To test whether microfilaments are specifically involved in the anchoring (but not localization) of Insc, Pros and Stau crescents, *in vitro* cultures were treated with colcemid to arrest neuroblasts in mitosis and accumulate Insc, Pros and Stau crescents, and then cytochalasin B or latrunculin B was added to disrupt microfilaments. Following addition of cytochalasin B, all three proteins fall off the cortex and fill the cytoplasm, with only a small fraction of the neuroblasts still showing asymmetric localization (Figure 4, Table 1). This effect is reversible. When cytochalasin B is washed out (leaving colcemid to maintain the metaphase block), all three proteins relocalize into asymmetric cortical crescents at the expected positions (Insc crescent opposite Pros and Stau crescents) and at the normal frequency (Figure 4, Table 1). When latrunculin B is used in the same experiment, microfilaments are totally disrupted and virtually all neuroblasts show cytoplasmic distribution of all three proteins, but there is very little recovery (Figure 4, Table 1). Lack of recovery is not due to cell death as determined by a live/dead cell-staining assay (see Materials and methods); it is

Table 1

The effect of cytoskeletal inhibitors on asymmetric protein localization in mitotic neuroblasts.

	Prospero				Staufen				Inscuteable			
Drug	Asym (%)	Ring (%)	Cyto (%)	*n*	Asym (%)	Ring (%)	Cyto (%)	*n*	Asym (%)	Ring (%)	Cyto (%)	*n*
WT	88	0	12	64	84	0	16	19	89	0	11	45
COL	92	0	8	131	89	1	10	71	87	0	13	38
CB	15	5	80	102	12	7	81	41	53	25	22	61
LB	0	0	100	106	0	0	100	50	10	82	8	50
COL+CB	10	20	70	124	9	15	76	80	20	9	72	46
CB REC	87	4	9	94	83	4	13	52	74	0	26	42
COL+LB	0	1	99	150	0	3	97	63	6	7	86	87
LB REC	5	20	75	153	4	17	79	72	10	9	81	81

WT, no drug treatment; COL, colcemid; CB, cytochalasin B; LB, latrunculin B; CB REC, washout of CB only following COL+CB treatment; LB REC, washout of LB only following COL+LB treatment (see Materials and methods for details). Asym, asymmetric cortical localization; ring, uniform cortical localization; cyto, cytoplasmic or undetectable; *n*, number of neuroblasts scored. All neuroblasts were scored at mitosis as determined by DNA staining.

probably due to the incomplete recovery of the microfilament cytoskeleton (Figure 3).

We conclude that the ability to anchor Insc, Pros and Stau at the apical or basal cortex is correlated with the integrity of the microfilament cytoskeleton. Neuroblasts with normal uniform cortical microfilaments (wild type, colcemid-treated or cytochalasin B recovery) show normal protein crescents; neuroblasts with abnormal punctate cortical microfilaments (cytochalasin B-treated or latrunculin B recovery) or no microfilaments (latrunculin B-treated) show virtually no protein crescents.

It has previously been reported that asymmetric localization of Pros and Numb does not require microfilaments [14]. In contrast, we show that Pros, Stau and Insc localization absolutely requires microfilaments. The previous study used 1 μg ml^{-1} cytochalasin B *in vivo*, conditions in which neuroblasts may be exposed to low and/or variable concentrations of the drug as a result of permeability problems [35]. Furthermore, cytochalasin B does not completely disrupt microfilaments. In our studies, all cells are exposed to a known concentration of cytochalasin B or latrunculin B; cytochalasin B partially and reversibly inhibits Pros, Stau and Insc localization, whereas the more effective microfilament inhibitor latrunculin B gives complete but irreversible loss of asymmetric protein localization.

We draw several conclusions from our drug treatment experiments. First, microfilaments are essential for anchoring Insc, Pros and Stau asymmetrically to the neuroblast cortex. Microfilaments may also be involved in protein localization, but the simplest hypothesis is that 'diffusion plus anchoring' is sufficient for asymmetric protein localization. Second, Pros and Stau are always tightly co-localized when at the cell cortex, and always identically affected by drug treatments, suggesting that Pros and Stau are part of a single protein complex that is uniformly affected by loss of microfilaments. This complex is likely to contain Mira, which is required for Pros localization and interacts with Pros directly [20,21]. Third, there is a hierarchy of sensitivity to loss of microfilaments: Pros/Stau > Insc > apical–basal polarity cues. Pros and Stau are the most sensitive to microfilament disruption, indicating that loss of microfilaments affects Pros/Stau anchoring directly. Insc is more resistant to microfilament disruption, but still requires microfilaments for apical anchoring. Finally, apical/basal polarity cues (that regulate Insc, Pros and Stau localization) are most resistant to microfilament disruption, and may be independent of microfilaments. Partial microfilament disruption by cytochalasin B results in loss of Insc, Pros and Stau crescents; apical–basal polarity cues are not affected, as seen by recovery of crescents following washout of cytochalasin B. Complete disruption of microfilaments results in the non-recoverable loss of protein crescents, which could be due to either the incomplete recovery of microfilaments or the loss of apical–basal polarity cues; we cannot distinguish between these possibilities. Fourth, Insc can be anchored at the neuroblast cortex by either microtubules or microfilaments. In the absence of both microtubules and microfilaments, Insc is cytoplasmic; in the absence of microfilaments alone, Insc is uniformly cortical, revealing a role for microtubules in stabilizing Insc at the cortex; in the absence of microtubules

alone, Insc forms an asymmetric cortical crescent, perhaps due to diffusion plus microfilament-dependent asymmetric anchoring.

Conclusions

Extrinsic cues are required for localization of Insc, Pros and Stau in interphase neuroblasts, but intrinsic cues are sufficient for unequal neuroblast cytokinesis and the asymmetric localization of Insc (apically) and Pros and Stau (basally) during neuroblast mitosis. Thus, interphase protein localization appears unnecessary for subsequent protein localization at mitosis. We find that microfilaments, but not microtubules, are essential for the asymmetric anchoring (and perhaps localization) of Insc to the apical cortex and Pros and Stau to the basal cortex of mitotic neuroblasts. A simple model is that Insc, Pros and Stau proteins diffuse to the cortex and are asymmetrically anchored at apical or basal sites by a microfilament-dependent mechanism. Our results highlight the central role of microfilaments in the asymmetric localization of proteins and RNAs across a wide spectrum of organisms, including vertebrates, fungi, plants and *C. elegans* and in *Drosophila* oogenesis and neurogenesis [8,32,36–39].

Materials and methods

In vitro *neuroblast culture*

Cell cultures were prepared from 4–5 h embryos. Dechorionated embryos were rinsed for 5 min in 95% ethanol and then equilibrated in culture medium (CM, Schneider's insect medium plus 2% fetal calf serum, Sigma). Embryos were homogenized in CM by six to eight strokes of a loose-fitting dounce. The cell suspension was filtered through a 30 μm Nitex mesh, and cells were pelleted in a clinical centrifuge at 4°C (setting 5, IEC). The cell pellet was washed twice by pouring off the supernatant and gently triturating the pellet in fresh CM. After the final wash, the dissociated cell suspension was plated in 0.5 ml on clean glass coverslips. The density of cells was empirically determined to achieve isolated cells with some small clusters. Cells were allowed to adhere to the coverslips for 30 min prior to the addition of 1.5 ml CM with or without drugs. Cultures were grown for 2 h at 25°C, during which time untreated neuroblasts complete one or two cell divisions. Cultures were fixed for 15 min in 4% methanol-free formaldehyde in PEM (0.1 M PIPES, 1 mM EGTA, 1 mM $MgCl_2$ pH 7). Neuroblasts were identified as large cells adjacent to small cells with nuclear Pros (putative GMCs); cells in large clusters were not counted. A small proportion of these cells could be asymmetrically dividing precursors of the gut, peripheral nervous system or procephalic region.

Drug treatments

All drug treatments were carried out in CM. Single drug treatments were as follows: 5 μg ml^{-1} colcemid (Sigma) added 30 min after cell plating and incubated 1 or 2 h; 5 μg ml^{-1} cytochalasin B (Sigma) added 2.5 h after cell plating and incubated 30 min; 1 μg ml^{-1} latrunculin B (Calbiochem) added 2.5 h after cell plating and incubated 5 min. Double drug treatments were as follows: 5 μg ml^{-1} colcemid (Sigma) added 30 min after cell plating and incubated 2 h, followed by addition of 5 μg ml^{-1} cytochalasin B for 30 min or 1 μg ml^{-1} Latrunculin B for 5 min. Recovery experiments were done exactly as double drug experiments, except that after cytochalasin B or latrunculin B treatment the cultures were washed with CM containing 5 μg ml^{-1} colcemid to remove the microfilament inhibitors and cultured an additional 40 min in CM containing colcemid.

Live/dead cell assay

Living cultures were incubated with the DNA stain TOTO-1 and examined by epifluorescence: dead cells show fluorescent staining of the DNA, whereas living cells are unstained. None of the drug treatments resulted in a detectable increase in cell death (data not shown).

Antibody staining

Antibody staining was done essentially as described [13,40] using rabbit anti-Insc (1:2000), mouse anti-Pros MR1A (1:4), and rabbit anti-Staufen (1:1000) primary antibodies overnight at 4°C followed by rhodamine- or Cy5-conjugated secondary antibodies (1:400; Jackson Immunoresearch) for 1 h at room temperature. Double labeling was done by sequential application of antibodies. DNA was detected using 1 mg ml^{-1} sonicated *para*-phenylenediamine in 90% glycerol [41]. Microfilaments were visualized using fluorescein isothiocyanate (FITC)-conjugated phalloidin (1:200; Molecular Probes). Images were collected on a BioRad confocal microscope and processed using Photoshop (Adobe).

Defining the apical–basal axis in vitro

The apical–basal axis of neuroblasts *in vitro* was defined using two criteria: first, during anaphase–telophase, it is clear which daughter cell is smaller, and this side of the neuroblast is defined as basal (*in vivo* the smaller GMC always buds off from the basal side of the neuro-blast); second, since Insc is always apical and Pros and Stau basal in anaphase–telophase neuroblasts, we define the site of Insc as apical and the site of Pros and Stau as basal in prophase–metaphase neuroblasts.

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